SCALE UP OF BIOPOLYMER (PHB) FERMENTATION FROM 500 mL SHAKE FLASKS TO 2L STIRRED TANK FERMENTOR

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I declare that this thesis entitled “Scale up of Biopolymer (PHB) Fermentation from 500 mL Shake Flasks to 2L Stirred Tank Fermentor” is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : …….....
Name : AIMI SALMA BINTI AWALLUDIN
Date : ……

To my beloved parents, brothers and sisters

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ABSTRACT

This study is about the scale up of biopolymer fermentation from 500 mL shake flasks to 2L fermentor. The purpose of this project is to determine air flow rate at 2L fermentor which gives the same k_La and ka_p as produced in shake flasks. The rationale of using the k_La value is to ensure a certain mass transfer capability in order to cope with the oxygen demand of the culture, thus being an important scale-up factor. Both values were derived by fitting the dissolved oxygen tension (DOT) versus time data into The Fibonacci Min Search (Fminsearch) method. Two values, which are 0.2960 for volumetric mass transfer coefficient for oxygen (k_La) and 0.0220 for the electrode mass transfer coefficient (ka_p) were obtained at small scale. These values are required to be duplicated in the larger scale. It was done by using a fixed agitation rate of 200 rpm and a manipulated aeration rate which is 1L/min,1.5L/min,2L/min and 1.75L/min. The most comparable k_La and ka_p values obtained from the trial are at 1.75L/min. This aeration rate will be used in the 2L fermentor in order to investigate the production of poly-β-hydroxybutyrate (PHB). Fermentation is run at both scales to compare the glucose, biomass and PHB profile. From the experiment and calculation, the maximum concentration of PHB is achieve at the 36^{th} hours, which is 1.415g/L for 500 mL shake flasks, and 2.17g/L for 2L fermentor. The cell dry mass obtained at the optimum harvesting time is 6.065 g/L for both scales.

ABSTRAK

Kajian ini adalah mengenai menskala naik fermentasi biopolimer dari 500 mL kelalang goncang ke 2L tangki teraduk. Tujuan utama projek ini adalah untuk mencari kadar aliran udara pada skala 2L tangki teraduk yang memberi nilai k_La dan ka_p yang sama sebagaimana terhasil dari kelalang goncang. Rasionalnya, pengunaan nilai k_La adalah untuk memastikan kemampuan pekali pemindahan jisim yang berupaya memenuhi keperluan oxygen kultur lantas menjadi faktor penting untuk menskala naik. Kedua-dua nilai dihasilkan dengan memasukkan data tekanan oksigen terlarut (DOT) menentang masa pada kaedah The Fibonacci Min Search (Fminsearch). Dua nilai iaitu 0.2960 untuk pekali pemindahan jisim bagi oksigen dan 0.0220 bagi pekali pemindahan jisim bagi elektrod terhasil pada skala kecil. Nilai-nilai ini adalah perlu untuk diduplikasi pada skala yang lebih besar. Ia dihasilkan dengan menggunakan kadar pengadukan(rpm) yang tetap iaitu 200 dan kadar aliran udara yang dimanipulasikan iaitu 1L/min,1.5L/min,2L/min and 1.75L/min. Nilai k_La dan ka_p terhampir didapati dari cubaan adalah pada kadar aliran udara 1.75L/min. Kadar aliran udara ini akan digunakan pada 2L tangki teraduk untuk mengkaji penghasilan poly-β-hydroxybutyrate (PHB). Fermentasi dilakukan pada kedua skala untuk membandingkan profil gula, jisim kering sel dan PHB. Menerusi eksperimen dan pengiraan, nilai tertinggi bagi kepekatan PHB didapati pada jam ke 36, adalah 1.415g/L untuk 500 mL kelalang goncang, dan 2.17g/L bagi 2L tangki teraduk. Berat kering sel terhasil pada masa tuaian optimum adalah 6.065 g/L pada kedua-dua skala.

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LIST OF ABBREVIATIONS

C*	-	Dissolved oxygen concentration.
DCW	-	Dry cell weight
DO	-	Dissolved oxygen
DOT	-	Dissolved oxygen tension
ka_p	-	Electrode mass transfer coefficient
k_La	-	Volumetric mass transfer coefficient for oxygen
NGY	-	Nutrient Glucose Yeast
OTR	-	Oxygen transfer rate
OUR	-	Oxygen uptake rate
PHB	-	Poly ß hydroxyl butyrate
rpm	-	Rotation per minute
t	-	Time
YR(t)	-	The value of dissolved oxygen from calculation (theory)

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

The problem concerning solid waste management and global environment have formed significant interest in the development of biodegradable plastic in recent times. The intrinsic qualities of durability and resistance to degradation over the last two decades have been increasingly regarded as a source of environmental and waste management problem emanating from plastic materials (Poirier *et al*, 1999).

There is an urgent need to address the problem of improving productivity and yield of Poly-β-hydroxybutyrate (PHB) production through fermentation so that it can provide a viable alternative and economically compared to the production of conventional plastic material.

On the lab scale, fermenting of PHB had brought about promises to the mass production of biodegradable plastic. However, there are still issues and obstacles that require research to be carried out before such becomes a reality. Badly needed are viable solutions to the production of biodegradable plastic for the use of mankind today.

1.2 Problem Statement

Environmental concerns are the biggest threat to the conventional plastics industry today. Among the issues are the releases of greenhouse gas, toxic pollutants, and non-biodegradable landfill impact. This is the result of the irresponsible disposal of petroleum and petroleum-based plastics.

Because of the environmental problem cause by polymer, numbers of research are done to find an alternative ways to reduce the use of conventional plastic. One of the approaches is by producing biodegradable plastic or biopolymer.

Simultaneously, several factors inhibit the large scale of biopolymer production and commercialization. These include the high cost of production in terms of media substrate raw material such as glucose, the extraction method, and the market price for PHB based plastic, are higher than polymer from petrochemical product.

Usually, the productivity of the desired product is high in small scale, and will be gradually reduced as the scale is enlarged because of the complexity of fermentation process. In scale up process, besides the development of inoculums and medium sterilization, the aeration and agitation presence in culture are also some of the arising problem.

Ideally, oxygen transfer rate should be measured and the basis of constant volumetric transfer coefficient for oxygen (k_La) is used in order to scale up. Another parameter that also contributes towards the obtaining of the aeration rate needed, would be the electrode mass transfer coefficient (ka_p). In scale up process, both parameters should be in positive value. The problem arise when previous research dealing with this process obtaining the negative ka_p.

1.3 Objective

To scale up the biopolymer (PHB) fermentation from 500mL shake flask to 2L stirred tank fermentor.

1.4 Scopes of the Research Work

In this study, the scopes of research are focusing on several aspects which are:

1. To determine air flow rate at 2L fermentor which gives the same k_La and kap produce by shake flask with a positive value.
2. To scale up the fermentation at 2L and comparing the glucose, biomass and PHB profile.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Plastic Industry

2.1.1 Background of plastic

Plastic literally means "changeable" and it refers to any natural and synthetic materials that can be shaped when soft and then hardened. Today, the plastic industry is heavily integrated with the oil industry. In fact a popular view is that it would not be able to produce plastics if oil were not available. This is very different from the situation 40-50 years ago when the plastic industry was being described as a 'scavenger of raw material' (Brydson, 1999).

However, conventional plastic produced from the petroleum based sources are causing multitude problems and concerns because such products cannot easily degrade. It was cited that non-degradable plastics accumulate in the environment at a rate of more than 25 million tones per year (Lee, 1996). Newer biodegradable plastic production with the aid of microorganisms is thus urgently needed.

2.1.2 Development Biodegradable Polymer (Biopolymer)

Biopolymers are polymers that can be synthesized from living organism. Examples of input materials that can be used to produce biopolymer are starch, sugar, cellulose or other synthetic materials.

Biopolymers may be defined as products which are based on renewable agricultural or biomass feedstock, capable of behaving like conventional plastics in production and utilization, but degradable through microbial processes upon disposal. It is this progressive development of biopolymers which has led to a surging interest of a plastic and composite industry based on biological materials (Mohanty *et al*, 2003).

Some of the biodegradable plastic materials under development include poly-hydroxyl-alkanoates (PHAs), polylactides, aliphatic polyesters, polysaccharides, and copolymers and blends of starch and polypropylene (Lee *et al*, 1996).

2.1.3 Rational behind Biopolymer

Recently, a large scale production of Poly-β-hydroxybutyrate (PHB) by bacteria has become a subject of increasing interest. PHB is a useful biodegradable polymer which can be used as a thermoplastic (Byrom, 1987; Holmes, 1985; Doi 1990).

Biopolymers are possible alternatives to the traditional, non-biodegradable petrochemical derived polymers. In terms of molecular weight, brittleness, stiffness and glass transition temperature, the PHB homopolymer is comparable to some of the more common petrochemical-derived thermoplastics, such as polypropylene (Barham, 1990).

2.2 Poly-β-hydroxybutyrate (PHB)

2.2.1 Introduction

Poly-β-hydroxybutyrate (PHB) belongs to the class of biodegradable plastics PHAs. PHB was first among the family of PHAs to be detected by Lemoigne in 1926 as a constituent of bacterium *Bacillus megaterium* (Lemoigne, 1926). Approximately 150 different hydroxyalkanoic acids are at present known as constituents of these bacterial storage polyesters (Steinbüchel and Valentin 1995).

Polyhydroxyalkanoates (PHAs), a family of bacterial polyesters, are formed and accumulated by various bacterial species under unbalanced growth conditions. PHAs have thermomechanical properties similar to synthetic polymers such as polypropylene, but are truly biodegradable in the environment (Lee *et al*, 1996).

The molecular structure of PHB are describes in Figure 2.1. PHB act as an energy storage facility, and are developed when the bacteria's surroundings include excess carbon, and a deficiency of another nutrient.

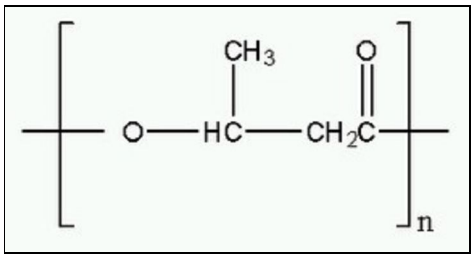

Figure 2.1: Structure of PHB

2.2.2 Synthesis route / Production of PHB

PHB are produced by many genera of bacteria as inclusion bodies to serve as carbon source and electron sink. PHB is synthesized from acetyl-CoA produce by the bacteria in sequential action of three enzymes. 3-ketothiolase (phbA gene) catalyses the formation of a carbon-carbon bond by condensation of two acetyl-CoA (Masamune *et al*, 1989)

NADPH dependent acetoacetyl-CoA reductase (phbB gene) catalyses the stereoselective reduction of acetoacetyl-CoA formed in the first reaction to R-3-hydroxybutyryl CoA. The third reaction of this pathway is catalyzed by the enzyme PHB synthase (phbC gene) that catalyzes the polymerization of R-3- hydroxybutyryl-CoA to form PHB. The EC number is yet to be assigned to PHA synthase (Steinbüchel and Schlegel 1991, Belova *et al*. 1997). Figure 2.2 showed the biosynthetic pathway of PHB from acetyl-CoA

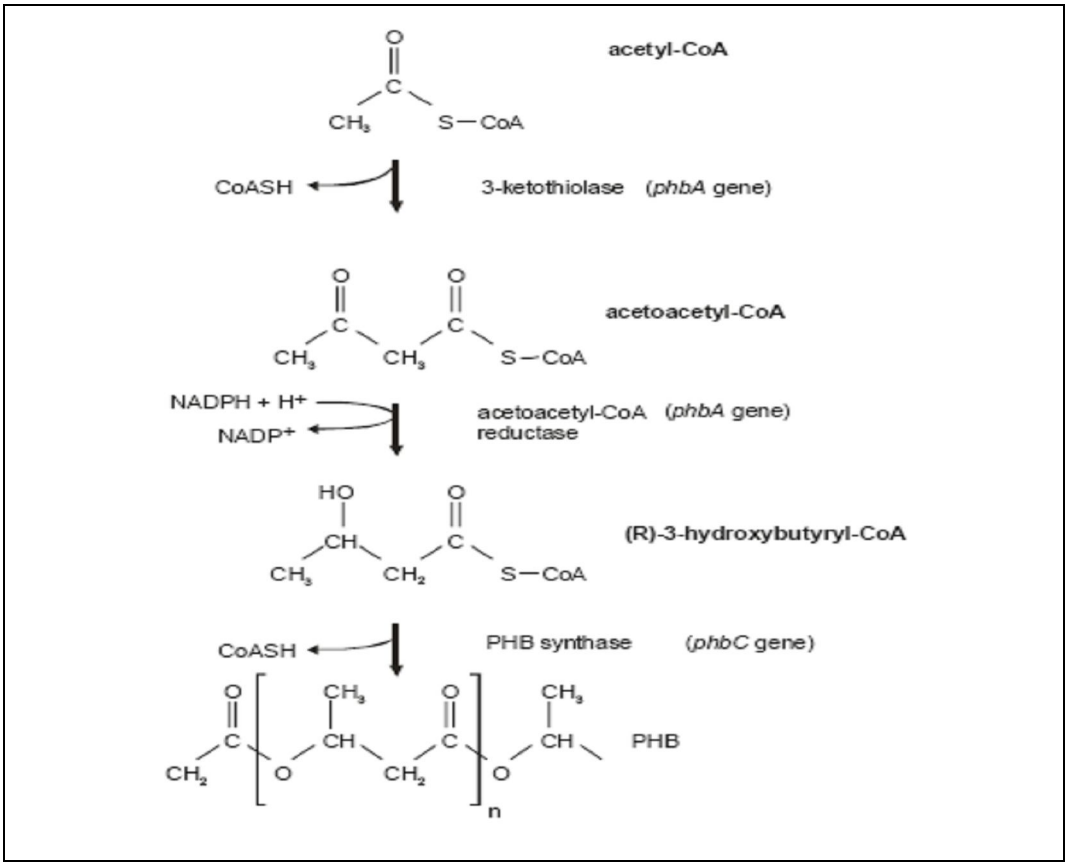

Figure 2.2: Biosynthetic pathway of PHB from acetyl-CoA (Taguchi et al.)

2.2.3 Application of PHB

Economic and technological barriers are the main concerns regarding large-scale microbial production of PHAs and poly-β-hydroxybutyrate (PHB). Byrom cited that large scale production of poly-β-hydroxybutyrate(PHB) by bacteria has become a subject of increasing interest (Byrom, 1897).

Applications focus in particular on packaging such as containers and films (Bucci and Tavares, 2005). It is also processes into toners for printing applications and adhesives for coating applications (Madison and Huisman, 1999*)*.

PHB could replace some of the more traditional, non biodegradable polymers. Polymer blends is expected to be more widely accepted. It is cited that such blends will greatly increase the spectrum of possible applications by expanding the range of available physical properties. PHB in combination with other biocompatible and nontoxic polymers would also have an enhanced scope in biomedical applications (Christi *et al*, 1999).

2.2.4 Advantages of PHB

The viability of microbial large-scale production of polyhydroxyalkanoates (PHAs) is dependent on the development of a low cost process that produces biodegradable plastics with properties similar or superior to petrochemical plastics. A shift emphasis in biomaterials engineering in recent years has moved the focus of attention from materials that will remain completely stable in the biological environment, to materials that will, in some way, alter their properties or biodegrade. poly-β-hydroxybutyrate (PHB) is polyester made by micro-organisms and is fully biodegradable.

The activities of these enzymes may vary and depend on the composition of the polymer and the environmental conditions. The degradation rate of a piece of PHB is typically in the order of a few months in anaerobic sewage to years in sea water (Madison and Huisman, 1999). Yet, ultraviolet light can accelerate the degradation of PHAs (Shangguan *et al*, 2006).

The main advantage in the medical field is that PHB is a biodegradable plastic which can be inserted into the human body and does not have to be removed again. It is also biocompatible as it is a product of cell metabolism and also 3-hydroxybutyric acid, the product of degradation which is normally present in blood concentrations between 0.3 and 1.3 $mmoll^{-1}$ (Zinn *et al*, 2001).

2.2.5 Disadvantages of PHB

There are some of disadvantages of using PHB as a plastic material since its tendency to be brittle. Apart from brittleness, price is also another drawback. The high price of commercial grade PHB- about 15-fold greater than comparable synthetic plastic limits its use to specialist niches. For example, Biopol, a copolymer of β-hydroxybutyric acid and of β-hydroxyvakeric acid produced by Ralstonia eutropha, sell about 17 times the price of synthetic plastic(Braunegg *et al*, 1998).

2.3 Fermentation of BioPolymer

2.3.1 Introduction to Fermentation

In its broadest sense, fermentation refers to any process by which large organic molecules are broken down to simpler molecules as the result of the action of microorganisms. The most familiar type of fermentation is the process by which sugars and starches are converted to alcohol by enzymes in yeasts. Normally, the

fermentor volume is usually filled to only 70–80% of its total capacity, to leave head space above the fermentation broth (Bailey and Ollis, 1986).

Fermentation is the use of microorganisms to break down organic substances in the absence of oxygen. Today, fermentation can be carried out with genetically engineered microorganisms, specially designed for the conditions under which fermentation takes place, and for the specific substance that is being broken down by the microorganisms. In this study, fermentation is the process used to produce PHB.

2.3.2 Microorganism

For fermentation to take place, the microorganism that is used to produce PHB in this study is *Cupriavidus necator* (also known as *Ralstonia eutropha* or *Alcaligenes eutrophus*). The reason for choosing this microorganism is because it had been found out that *Alcaligenes eutrophus* is the prime PHB producer *(*Doi *et al*, 1987*)*.

Ralstonia Europha (formerly *Alcaligenes eutrophus*) is the most extensively studied bacterium in both basic and applied research on the formation of PHAs. This species can accumulate PHAs up to 80% (wt.) of dry cell mass using various carbon sources including carbohydrates, alcohols and organic acids (Anderson and Dawes, 1990).

Alcaligenes eutrophus can use inexpensive carbon sources, which is important in industrial scale production. The organisms show differences in their growth and polymer production conditions but they were chosen because of their high polymer production capacity. Another criterion for the selection is the ease of separation of the polymer from the cells.

2.4 Scale up of Biopolymer Fermentation

2.4.1 Introduction to scaling up

Scale up is the process whereby small scale production (several culture dishes) is transformed to a large scale production (a reactor of several liters). In other words, scale up is to perform an experiment in bulk, after the optimal conditions have been determined by a screening experiment. Both definitions referred to a process in which the data from an experimental scale operation is used in a larger scale unit for larger production.

The purpose of scaling up is to obtain the same product per volume in both small scale and large scale at the same time. The basis of constant volumetric transfer coefficient (k_La) of oxygen is used in order to scale up. During scale up, three major factors should be considered to eliminate problem that will arise which are inoculum's development, medium sterilization and aeration.

2.4.2 Important parameter in scale up processes

Biopolymer synthesis generally occurs only when the microorganism is grown aerobically and usually under non-limited oxygen conditions, a polymer with higher molecular weight is produced (Sutherland, 1998) The supply of oxygen (OTR) can be the controlling step in industrial bioprocesses, scale-up of aerobic biosynthesis systems (Al-Masry, 1999, Elibol and Ozer, 2000).

The OTR value depends on the air flow rate, the stirrer speed, mixing, etc. On the other hand, the OUR is limited by increase in viscosity resulting from polymeric property (Çalik *et al.*, 2000). Oxygen transfer can play an important role since it is often the limiting factor in order to obtain the appropriate volumetric oxygen transfer